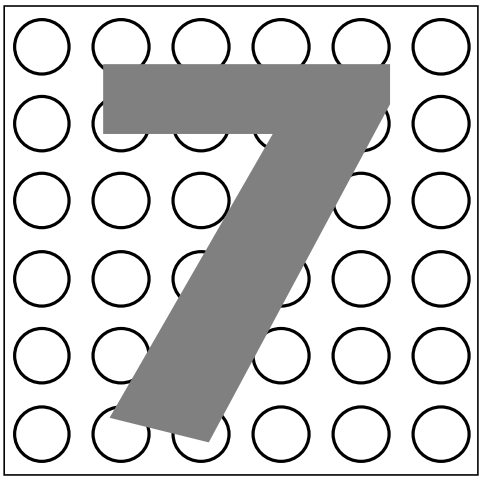
Human and rat organ slices

de Kanter, Ruben



A new technique to prepare precision-cut slices from small intestine and colon

Ruben de Kanter
Annemarie Tuin
Annelies Draaisma
Marina de Jager
Inge de Graaf
Dirk Meijer
Geny Groothuis

Groningen University Institute for Drug Exploration, Department of Pharmacokinetics and Drug Delivery, Groningen, Netherlands (RdK, AT, AD, MdJ, DM, GG)
Solvay Pharmaceuticals, Drug Safety Department, Weesp, Netherlands (IdG)

submitted

Abstract

A new technique was developed to prepare precision-cut slices from small intestine and colon with the object to study the biotransformation of drugs in these organs.
Rat intestinal slices were prepared in two different ways. In the first method, slices were punched out of the small intestine. In the second method slices were made from agarose filled and embedded intestines, using the Krumdieck tissue slicer. This method was also applied to colon tissue.
Viability of the slices was determined by intracellular ATP levels and morphology. Drug metabolizing activity was studied using lidocaine, testosterone and 7-ethoxycoumarin as phase I substrates, and 7–hydroxycoumarin as a phase II substrate.
Precision-cut slices made from agarose filled and embedded intestine showed better preserved ATP levels and morphology than slices that were punched out of the intestinal wall. Although the general crypt structure within the intestinal tissue was maintained, in parts of the slices prepared from small intestine (but not from colon), the villi were flattened after 3 h of incubation. The epithelium that was present in all preparations appeared to be intact. Morphology of agarose filled and embedded colon slices showed no signs of damage after incubation for 3 h. Intestinal slices showed high biotransformation activity.
It is concluded that preparing precision-cut 0.25 mm slices out of agarose filled and embedded intestine provides an improvement, compared with 'punched-out' slices, and that both intestinal and colon slices are useful preparations for in vitro biotransformation studies.

Introduction

The intestines can contribute significantly to the biotransformation of xenobiotica, because of their high content of drug metabolizing enzymes [161], since they contain transport systems to both the contraluminal and luminal domains of mucosal cells and because the small intestine is a major route for absorption of orally taken drugs.
Drug safety aspects such as the potential enzyme-induction, drug-drug interactions, species differences and prediction of the *in vivo* clearance and toxicity can, in principle, be studied using *in vitro* preparations, as has been proven with several liver-derived *in vitro* models in the past. One particular model, the model of precision-cut liver slices has been shown to be very valuable for all these aspects while at the same time, simple and convenient to use [171]. The slice technique has also been successfully applied to kidney and lung tissue in studies on drug metabolism and toxicity, as reviewed recently by us [63].
Analogous to the model of precision-cut liver, lung and kidney slices, our pres-

ent aim was to develop a convenient and simple *in vitro* slice-model of the intestine for biotransformation studies. Until now, slices from intestinal tissue have only been used rarely. Examples are studies on the metabolism of drugs in human intestinal slices [318,320,324] and the metabolic conversion of a cooked-food carcinogen in rat colon slices [189].

In a previous report, we showed that both rat and human small intestinal slices showed high biotransformation activities towards four model compounds, when compared with liver, lung or kidney slices [61]. In contrast to the observed high capacity of intestinal slices for biotransformation processes, ATP levels were relatively low in these slices that were punched out of the intestinal wall [61]. Therefore, we tried to improve the particular preparation technique in order to retain a better viability of the intestinal slices. We considered the possibility that the thickness of the punched out intestinal tissue was too large to allow efficient supply of oxygen and substrates to all the cells. Therefore, we prepared slices of 0.25 mm thickness, perpendicular to the intestinal wall, by filling and embedding the intestines in agarose and using the Krumdieck tissue slicer. Viability was assessed by measuring intracellular ATP levels and by examining histomorphology. Drug metabolizing activity was studied through the quantification of the formed metabolites from incubations with lidocaine, testosterone, 7-ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-HC) as model substrates. Furthermore, we applied the technique of filling and embedding intestines in agarose on large intestine (colon), and compared the metabolic capacity of slices from small intestine and colon.

Materials and Methods

Materials. The following compounds were obtained from the sources indicated: lidocaine from Centrachemie (Etten-Leur, the Netherlands); 16β-hydroxytestosterone from Steraloids (Newport, RI, USA); 7-EC from Fluka (Buchs, Germany); *N*-benzylimidazole, 2β-, 6β-, and 11β-hydroxytestosterone, testosterone, androstenedione, 7-HC, 7-hydroxycoumarin glucuronide (7-HC GLUC) and low melting agarose (type VII-A) from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands); Viaspan (Belzer UW) organ preservation solution from Barr Laboratories (Pomona, NY, USA); amphotericin B (Fungizone) and Williams' medium E (with Glutamax) from Invitrogen (Breda, the Netherlands). Monoethylglycinexylidide (MEGX) was a kind gift of AstraZeneca (Södertälje, Sweden), and 7-hydroxycoumarin sulphate (7-HC SULF) was a kind gift from Mr. P. Mutch, GlaxoWellcome (Herts, UK). All other chemicals were of analytical grade and were obtained from commercial sources.

Rat organ tissue. Animal handling was in accordance with the national laws on animal experimentation for the protection of vertebrate animals used for experimental and other scientific purposes. Male Wistar (HsdCpb:WU) rats (Harlan, Horst, the Netherlands) were housed in standard cages and had free access to food (standard 'RMH' chow, Hope Farms, Woerden, the Netherlands) and tap water. All experiments were performed with approval of the animal experimental regulatory authorities concerned. Rats (mean weight 390 g) were anaesthetised by isofurane and N_2O/O_2, and the intestines were excised and placed in Krebs-Henseleit buffer containing 10 mM Hepes and 25 mM glucose, pH=7.4, on ice. The intestines were flushed thoroughly with ice-cold Krebs-Henseleit buffer to remove the contents. Small pieces of each organ were cut off before the organs were excised and snap-frozen to determine *in vivo* ATP levels as described in 'viability of slices and punches'.

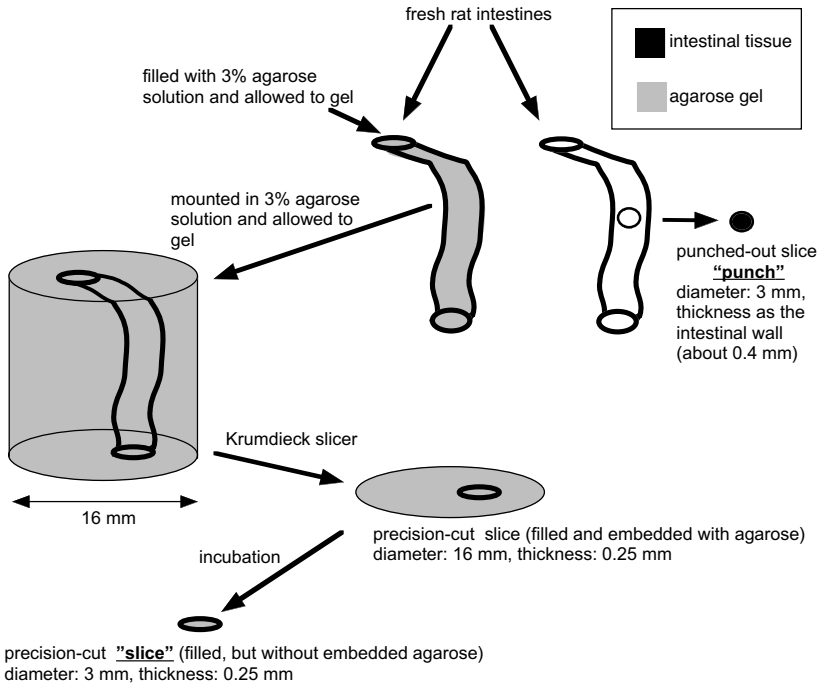


Figure 1. Schematic of the preparation of precision-cut intestinal slices using filled and embedded intestinal tissue and the Krumdieck tissue slicer.

Preparation of slices and punches (figure 1). Punches were made using skin biopsy cores (diameter 3 mm) from Stiefel (Sligo, Ireland), which were pushed through the intestinal wall after the muscle layer was carefully removed and the intestine was cut open.
To prepare agarose filled slices (figure 1), the intact intestines were first cut in 10 cm parts that were subsequently ligated on one side. These parts were than filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C and allowed to gel in ice-cold Krebs-Henseleit buffer. The agarose-filled intestines were cut in 1 cm parts and were embedded in the agarose solution at 37°C using the Tissue Embedding Unit from Alabama R&D (Munford, AL, USA) so that agarose gel cylinders with a diameter of 16 mm were formed. These cylinders were used to prepare precision-cut slices, with a diameter of 16 mm and a thickness of 0.25 mm, using a Krumdieck tissue slicer (Alabama R&D), pre-cooled and filled with oxygenated, ice-cold Krebs Henseleit buffer. Slices were stored in ice-cold Viaspan organ preservation solution until incubation (time gap between coring and incubation: max. 2 hr). To discern these agar embedded slices from punched-out slices, the latter will be further called 'punches' in this paper.
Incubation of slices and punches. Slices and punches were incubated in 3.2 ml Williams medium E, pre-warmed and gassed with 95% O_2/5% CO_2 and supplemented with glucose (final concentration 25 mM). To inhibit bacterial and fungus growth, gentamicin (50 µg/ml) and amphotericin B (Fungizone) (2.5 µg/ml) were added. These agents are poorly metabolized themselves and therefore assumed not to interfere with biotransformation enzymes. Slices were individually incubated in 6-well culture plates, which were placed in a plastic container and gassed with humidified 95% O_2/5% CO_2, and shaken back and forth (90 times/min) in a cabinet at 37°C. During incubation, the agarose gel surrounding the slices separated from the slices (without melting). As a result the diameter of the ring-like slices was 3 mm during incubation.
Viability of slices and punches. The viability of the slices and punches before and during incubation was determined by measurement of ATP-content of individual slices / punches. For this purpose, slices and punches were put in 1 ml 70% ethanol (v/v) con-

taining 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen. After storage at –80°C and homogenizing by sonication, ATP extracts were diluted ten times with 0.1 M Tris HCl/ 2 mM EDTA solution (pH 7.8) buffer to lower the ethanol concentration. The ATP content was measured using ATP Bioluminescence Assay Kit CLS II from Roche (Mannheim, Germany) and a 96-wells Lucy1 luminometer (Anthos, Durham, NC, USA).
To study histomorphology, slices and punches were fixed in 70% ethanol and stored at 4°C. After embedding in paraffin, cross-sections (5 µm) were made and stained by haematoxylin and eosin staining using standard procedures.
Metabolic activity of slices and punches. Metabolism of lidocaine (5 mM), testosterone (0.25 mM), 7-EC (0.5 mM) and 7-HC (0.5 mM) was studied by the addition of 32 µl stock-solutions in water (lidocaine), or methanol (testosterone, 7-EC and 7-HC) to 3.2 ml Williams' medium E. Metabolism was studied during 3 h of incubation. Medium samples (1 ml) of 7-EC incubations were acidified by adding 10 µl 2 M HCl, after sampling, in order to prevent spontaneous formation of metabolites that occurs when the pH of the incubation exceeds 8.0 [61]. Medium incubated for 3 h with substrates, but without slices, served as control and showed absence of spontaneous metabolite formation. Lidocaine, 7-EC and 7-HC medium samples were stored at –20°C until analysis. Preliminary experiments showed that no significant amounts of metabolites were retained in the slices (results not shown). After thawing, sodium azide was added to inhibit bacterial contamination (final concentration 0.1 mg/ml) and samples were analyzed using HPLC as described earlier for the MEGX metabolite of lidocaine [16] and for the metabolites of 7-EC and 7-HC [327]. For the quantification of 4-ethoxy-2-hydroxyphenyl propionic acid (EPPA), the standard curve of 7-EC was used because the absorption coefficients of EPPA and 7-EC are the same [61]. For all other metabolites we used authentic standards. For the metabolic biotransformation of testosterone, slices / punches and their incubation medium (inlcuding seperated agarose) were harvested together and homogenized using sonication to extract the metabolites from the slice. Control experiments showed that metabolites of testosterone were significantly retained in the slices (about 1.5 times the amount in medium, although metabolite profiles were similar) at the concentration used (results not shown). The homogenates were stored at –20°C. After thawing, 5 µl 11β-TOH, dissolved in methanol, was added as internal standard to 1 ml of the homogenate and 6 ml dichloromethane was then added. After removal of the water phase and protein aqueous interphase, the organic phase was evaporated and testosterone and its metabolites were dissolved in 130 µl 50% (v/v) methanol which was analyzed using HPLC as described earlier [313].
Protein content of slices and punches. After incubation, five slices or punches from each organ were taken and homogenized in their own incubation medium by sonication and diluted with 0.1 M NaOH. The protein content of the diluted homogenate was determined using Bio-Rad protein assay dye reagent (Bio-Rad, Munich, Germany) against a BSA standard curve. All metabolic activities and ATP levels are expressed per µg protein.

Results

Viability as judged by ATP content. To determine the relative viability in culture of intestine slices and punches, ATP levels were assessed *in vivo*, directly after preparation of the slices and punches, and after 1 and 3 h of incubation. In small intestinal punches with the muscle layer stripped off, ATP levels were retained at slightly higher levels than in punches with the muscle layer still attached, but this difference was not significant (figure 2). ATP levels in precision-cut slices that were made from agarose filled and embedded small intestine and colon were significantly higher than ATP levels in punches from these organs, at all time points ($p < 0.05$). For colon slices, this difference was greater than for small intestine slices (figure 2 and 3).

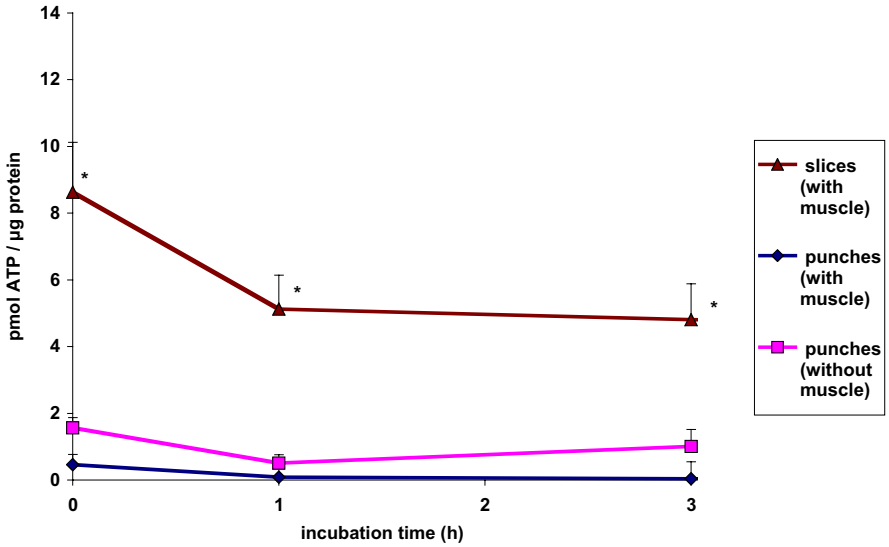


Figure 2. ATP levels in small intestine slices and punches during 3 h of incubation. Data are expressed as mean ± SEM from four independent experiments, in which the mean of three slices and punches was determined, at each time point.
* $p < 0.05$ vs. punches.

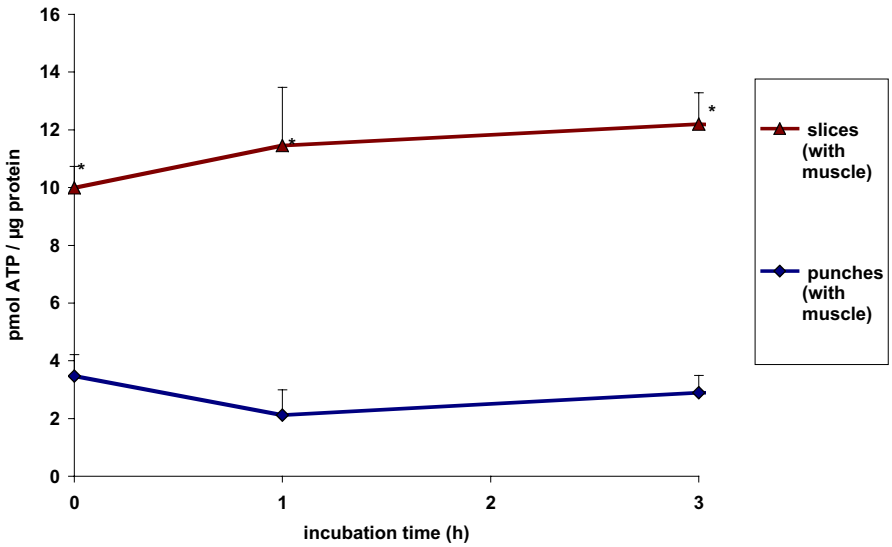


Figure 3. ATP levels in colon slices and punches during 3 h of incubation. Data are expressed as mean ± SEM from four independent experiments, in which the mean of three slices and punches was determined, at each time point.
* $p < 0.05$ vs. punches.

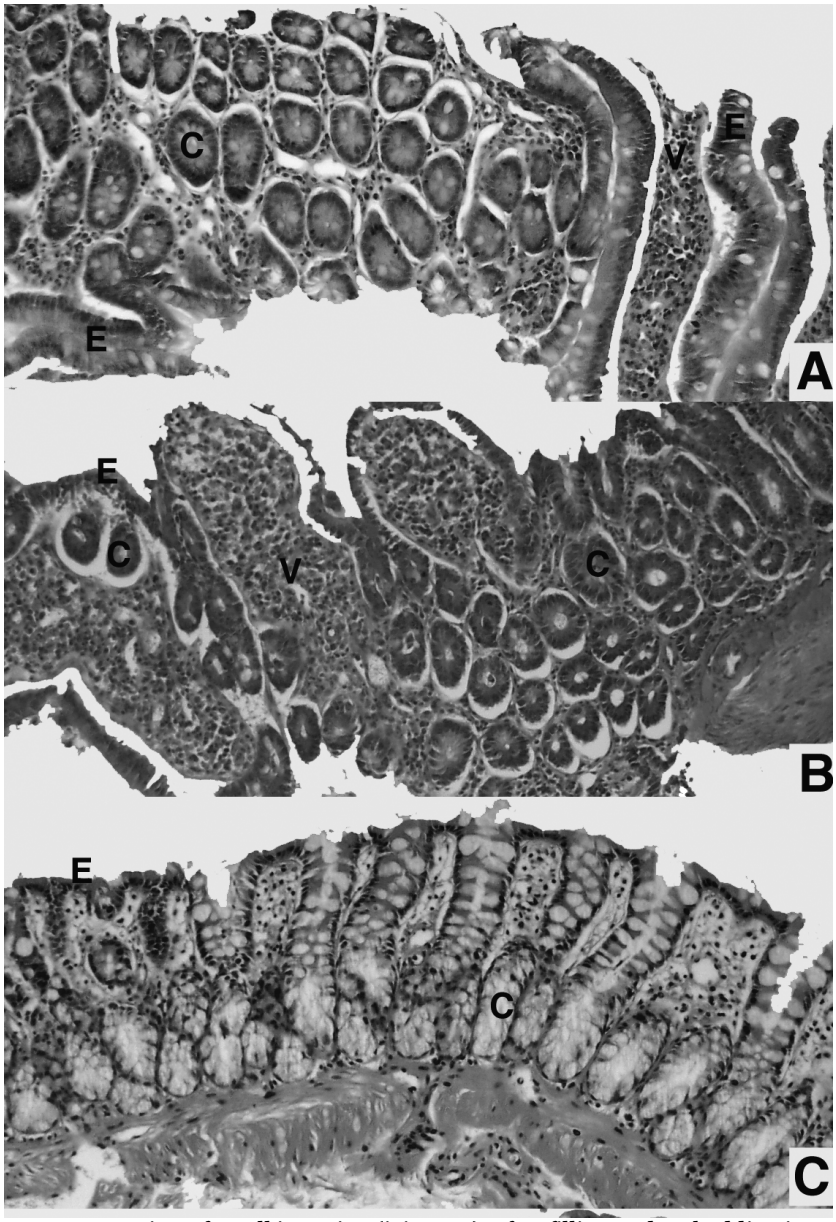


Figure 4A. A section of small intestine (jejunum), after filling and embedding in agarose and slicing, but not incubated. Inside the mucosal layer the crypts (C) are clearly visible. The villi (V) are pushed towards the slice due to the filling with agarose. The villi are enclosed by the epithelial layer of cells (E).

Figure 4B. A section of a precision-cut small intestinal slice after incubation for 3 h. Intact epithelium (E) in the crypts (C) and in the villi are visible, but marked flattening of the villi is observed, compared with non incubated slices.

Figure 4C. A section of colon, after filling and embedding in agarose and slicing, but not incubated. Crypts (C) the epithelial layer of cells (E) are clearly visible. Villi, that are characteristic for the small intestine, are absent.

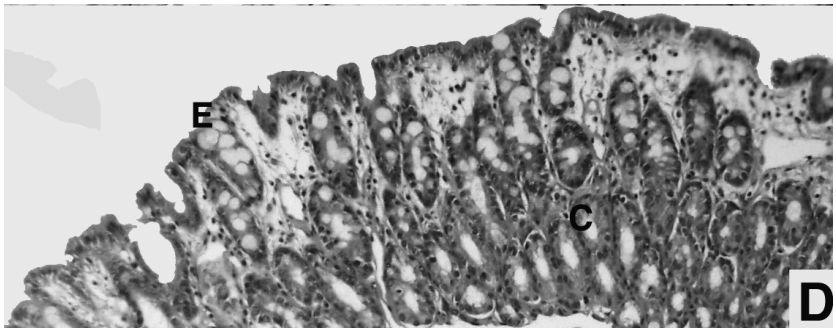


Figure 4D. Intact epithelium (E) in the crypts (C) and along the edges of the precision-cut colon slice, after incubation for 3 h.

ATP levels in precision-cut slices were about 2 to 4 fold higher than *in vivo* values, which were 1.79 ± 0.89 and 2.42 ± 0.74 pmol ATP / µg protein for small intestine and colon respectively (values ± SEM, n=7). We observed this phenomenon in earlier studies for the *in vivo* values of liver, lung, and kidney, compared with precision-cut slices from these organs [61], suggesting that *in vivo* ATP values are difficult to measure accurately, as was observed earlier [92]. In all preparations, ATP levels were decreased after 24 h of incubation to 40-60% of the values after 3 h of incubation (not shown).

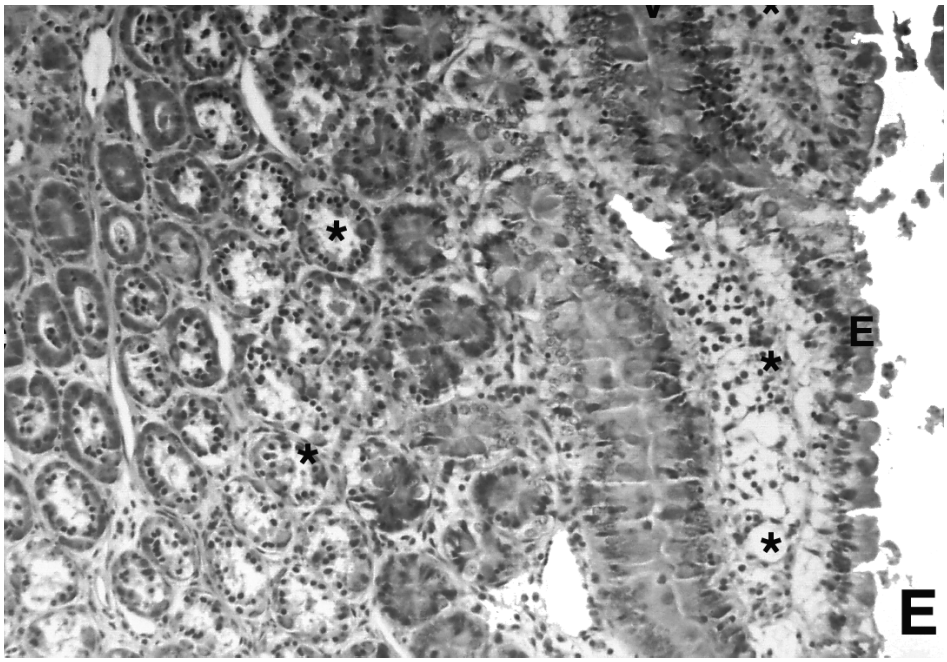


Figure 4E. Mucosal damage (*) observed in punches from the small intestine, after incubation for 3 h. The structure of the epithelium layer (E) is lost while only in the inner crypts (C) cells have a normal appearance, but not in the crypts towards the villi (*).

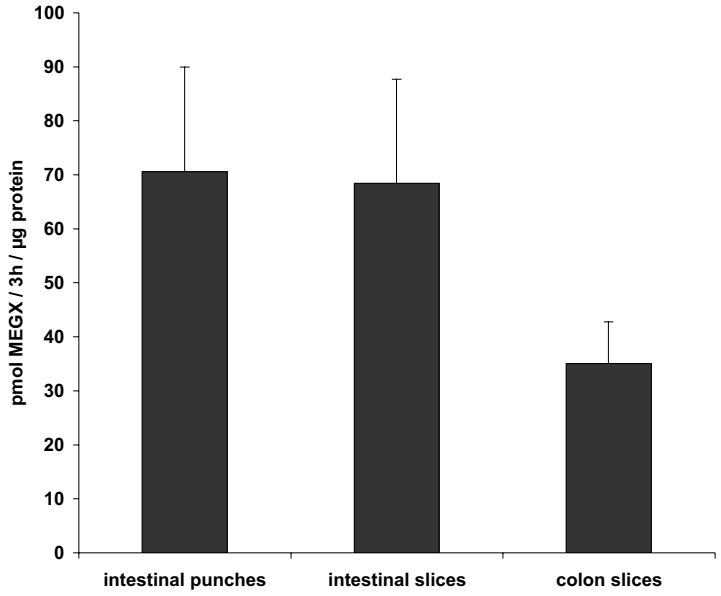


Figure 5. Lidocaine (5 mM) metabolism during 3 h incubation (data are means of four organs ± SEM and three slices / punches per organ).

Viability as judged by morphology. To get more insight in the viability of small intestine and colon slices and punches, microscopic examination was undertaken. All sections were stained with haematoxylin and eosin and viewed at a magnification of x 100.

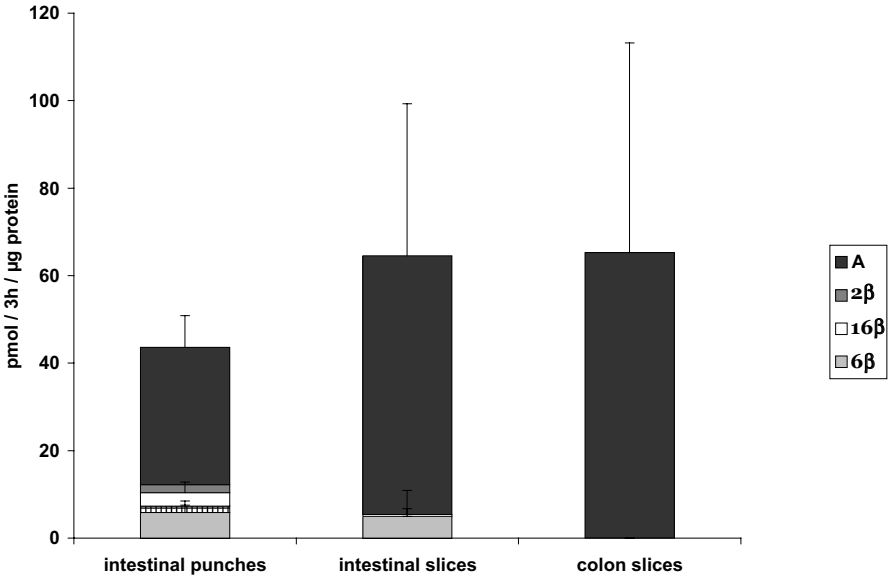


Figure 6. Testosterone (0.25 mM) metabolism towards androstenedione (A) and 2β-, 16β- and 6β-hydroxytestosterones (2β, 16β and 6β) during 3 h incubation (data are means of four organs ± SEM and three slices / punches per organ).

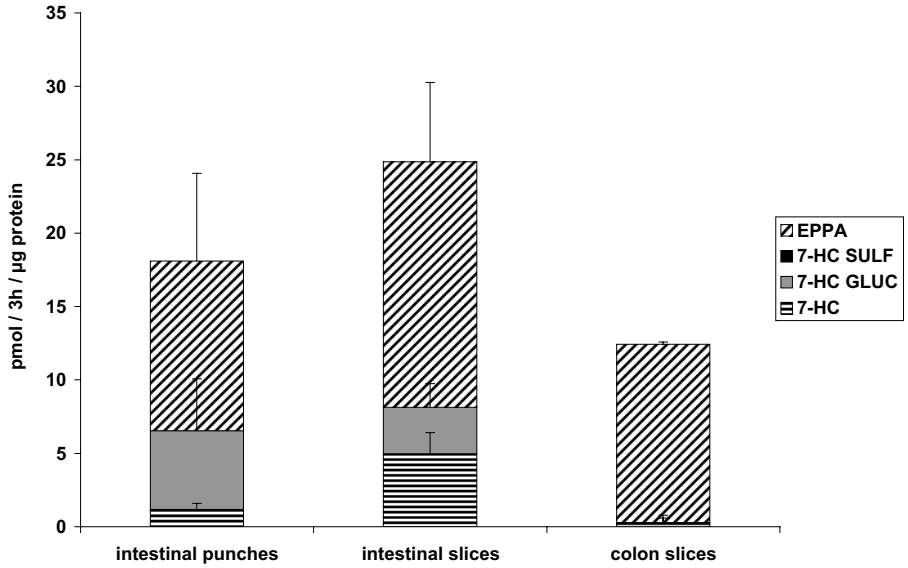


Figure 7. 7-EC (0.5 mM) metabolism during 3 h incubation towards 7-HC, EPPA, 7-HC GLUC and 7-HC SULF (data are means of four organs ± SEM and three slices / punches per organ).

As shown in figure 4e, punches from the small intestine showed abnormal morphology after 3 h of incubation. Extensive mucosal damage and villus destruction took place in these punches.
Precision-cut slices from agarose filled and embedded small intestine, incubated for 3 h (figure 4b) also showed flattening of the villi but general morphology is retained in the surface epithelium of villi.

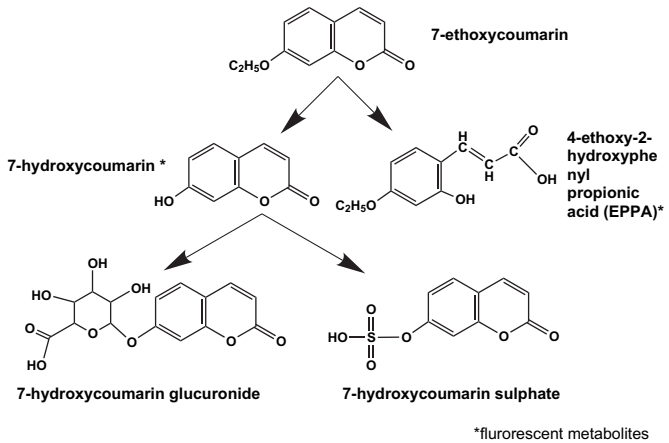


Figure 8. Metabolic pathway of 7-EC towards EPPA, 7-HC, 7-HC GLUC and 7-HC SULF.

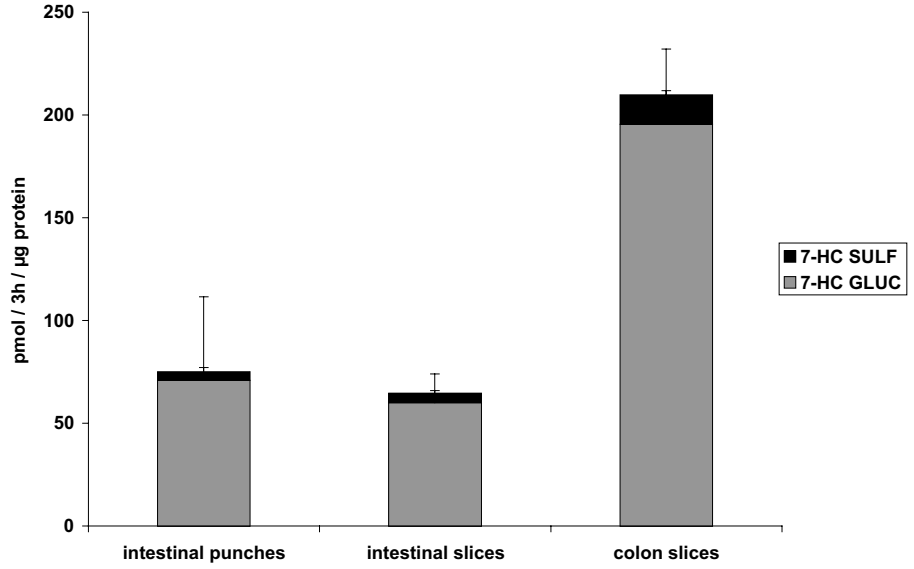


Figure 9. 7-HC (0.5 mM) metabolism during 3 h incubation towards 7-HC GLUC and 7-HC SULF (data are means of four organs ± SEM and three slices / punches per organ).

When the colon tissue photomicrographs were examined, intact surface epithelium seems to be present in precision-cut slices after 3 h of incubation (figure 4d), comparable to the situation normally present in the colon (figure 4c).

Metabolism. To reveal the metabolic capacity of intestinal slices and punches, incubations were performed with lidocaine, testosterone, 7-EC and 7-HC for the first 3 h of incubation.
In this study the *N*-deethylated metabolite, MEGX, of lidocaine was quantified. Punches had a similar capacity to form MEGX as precision-cut intestinal slices. Colon slices showed a two-third lower capacity to form MEGX, when compared with intestinal slices (figure 5).
It is shown in figure 6 that androstenedione was the main metabolite detected in testosterone incubations for both small intestine and colon. Punches had an average two-third lower capacity to form androstenedione compared with precision-cut intestinal slices. Colon slices showed the same capacity to form androstenedione if compared with intestinal slices. Colon slices did not form detectable amounts of hydroxylated testosterone metabolites and small intestinal preparations formed only minor amounts of 6β-hydroxytestosterone. Very minor amounts of 2β- and 16β-hydroxytestosterone were produced by punches of the small intestine, while these metabolites were not detected from incubations with precision-cut slices. Other hydroxylated metabolites could not be detected. The presence of less tissue in the precision-cut slice incubations is most likely the cause of undetectable amounts of other metabolites than

androstenedione, compared with punches that contain more intestinal tissue. Slices contained 0.16 mg of protein while punches had a protein content of 0.35 mg. Note that all metabolic data are normalized for protein content.
The metabolism of 7-EC is a commonly used parameter to investigate both phase I and integrated phase II metabolism *in vitro*. We showed earlier in extra-hepatic organs from both rat and man, that apart from the well described deethylation of 7-EC, hydrolysis of 7-EC takes place to form EPPA, as depicted in figure 8 [61]. As is shown in figure 7, it appeared that EPPA is the main metabolite in colon slices. Incubations in the presence of 1 mM cytochrome P450 inhibitor *N*-benzylimidazole showed similar formation of EPPA by intestinal slices, suggesting that cytochrome P450 is not involved in EPPA formation.
When small intestinal punches and slices were compared, it appeared that slices show more 7-HC and EPPA formation from 7-EC. However, less 7-HC is glucuronidated towards 7-HC GLUC by slices compared with punches from the small intestine. In colon slices, EPPA was the only detectable metabolite from incubations with 7-EC.
In figure 9 the conjugation (phase II) capacity of intestinal slices and punches is shown, using 7-HC as a model substrate. The main conjugated metabolite formed is the glucuronide conjugate, but also the 7-HC sulphate is formed by all intestinal preparations (see figure 9). Colon slices have a two-fold higher capacity to form 7-HC GLUC compared with intestinal slices. No differences between the different preparations of small intestine were observed.

Discussion

The aim of the present study was to develop a method for the preparation of slices from the intestines that retain proper viability. Previous reports [189,318,324] in which intestinal slices have been used, gave almost no details about their viability. As far as we knew, morphology data of intestinal slices have not been shown before.
We show that ATP levels were higher in punches from the small intestine after stripping off the muscle layer. A possible reason could be that diffusion of oxygen and nutrients is facilitated by removing the muscle layer, although it may also damage the punch. However, the thickness of the intestinal wall itself, which is about 0.4 mm in the rat (own observation), may still be a limiting factor for diffusion into the inner cell layers.
To overcome this problem, we have set up a method to prepare slices from agarose filled and agarose embedded small intestine, resulting in slices of only 0.25 mm thickness. This is thin enough to allow diffusion of oxygen and substrates to the inner cell layers. Indeed, we show here that both ATP levels and morphology in precision-cut slices from agarose filled and embedded small intestine were better preserved than in punches. Therefore, we conclude that

agarose filled and embedded intestine slices are an improved preparation compared with punched out preparations. However, morphologic examination showed flattening of the villi in the small intestinal slices after 3 h of incubation (figure 4b). In spite of this, the remaining cells in the slices showed normal morphology. Apparently, the villi are stripped off from the slices during incubation. In spite of these observations, intestinal slices show considerable biotransformation activity with regard to the four model substrates, exhibiting several metabolic routes as deethylation, oxidation, hydrolysis, as well as glucuronide and sulphate conjugation. These reactions are considered to take place in the epithelial mucosal cell layer [150]. It remains to be investigated if the epithelial cells that were still present in the slice fully account for the metabolic activity, or that the villi that are partly stripped off from the slices and remain intact in the incubations, may also contribute to the biotransformation activity. In any case, we conclude that these slices were useful for biotransformation studies because they show a relatively high metabolizing activity (for discussion, see below), apart from the better appearance and ATP content. Since we did not directly measure metabolic performance of intestinal tissues *in vivo*, it remains to be studied whether the observed activity is a good representation of the *in vivo* situation. It is not excluded that the damage of the intestines, as was observed by histomorphology, may affect the biotransformation activity in the slices.

Lidocaine deethylation towards MEGX is considered to be a result of cytochrome P450 activity in both man and rat [145]. We observed that MEGX formation took place in both small intestine and colon slices. This is in contrast with the earlier observation that colon punches lacked cytochrome P450 activity [189]. This might be either due to the improved preparation technique of colon slices used in the present study, or the possibility that MEGX formation in the colon is catalyzed by other enzymes than cytochrome P450 alone.

Earlier, testosterone metabolism in enterocytes was described to result in androstenedione formation (which is an oxidation reaction) *in vivo*, but *in vitro* reduction towards dihydroxytestosterone was favored [91], possible due to the relative hypoxic circumstances *in vitro*. Here we show that oxidative metabolism of testosterone takes place, producing androstenedione. This is in agreement with *in vivo* results in the rat [91]. In rat liver androstenedione formation is known to be catalyzed both by cytochrome P450 [347], and by 17β–hydroxysteroid oxidoreductase [91]. Because of the low cytochrome P450 content in the colon [189], 17β–hydroxysteroid oxidoreductase may be more likely to be involved in androstenedione formation.

Regarding the metabolism of 7-EC, we observed that both small intestine and colon slices mainly hydrolyze 7-EC to produce EPPA. Earlier, biotransformation of 7-EC has been studied in the isolated, perfused intestinal loop [2], isolated enterocytes [33,125] and microsomes from both small intestine [33] and colon [153]. These authors all reported the formation of 7-HC, in contrast to the present observation that EPPA is formed as the main metabolite. These authors used flu-

orometry to quantify the formation of 7-HC. Since the fact that EPPA shows the same fluorescence properties as 7-HC (unpublished observation), the reported fluorescence after incubation with 7-EC may actually be caused by EPPA instead of 7-HC (figure 8). In isolated intestinal cells of the rat the V_{max} of total 7-ethoxycoumarin metabolism was reported to be about 2.4 nmol/min/g intestine [34], which is much lower than to the total formation of 7-ethoxycoumarin metabolites in slices, being 17 nmol/min/g intestine [using a protein content of 12.5%, see 61]. A reason for this difference might lay in the possible selection of a sub-set of cells during the isolation procedure of intestinal cells together with the relatively low yield of viable isolated intestinal cells.

All intestinal preparations showed extensive metabolism of 7-HC. Conjugation of 7-HC was observed to be higher in colon slices than slices and punches from the small intestine, which is in agreement with the higher reported content of conjugation enzymes in the colon [189]. When normalized for protein content, colon slices appear to have even a higher capacity to conjugate 7-HC than liver slices [61]. The rates of 7-hydroxycoumarin glucuronide and sulphate formation, being 40 and 3.2 nmol/min/g small intestine [using a protein content of 12.5%, see 61] in rat intestinal slices (figure 9), are also higher than the reported V_{max} values of about 14.4 and 1.8 nmol/min/g intestine for 7-hydroxycoumarin glucuronidation and sulphation, respectively, as determined in isolated intestinal endothelial cells from the rat [160]. Like the difference in 7-EC metabolism, the improved viability of the slices together with the relatively low yield of viable isolated intestinal cells can be en explanation of this quantitative difference in rates of metabolism.

Recently, this method of preparing intestine slices by filling and embedding in agarose has also been applied to study biotransformation of three candidate drugs in the rat, using *in vivo*, and *in vitro* methods [60]. It appeared that the small intestine slice preparation was a proper model because they formed large amounts of a particular metabolite that was also the main metabolite observed *in vivo*. In contrast, this metabolite was only slightly formed by liver slices and isolated hepatocytes, and was not formed at all in lung or kidney slices, S9-mix, or microsomes from the liver [60].

Precision-cut intestine slices represent an improved *in vitro* technique compared with intestinal punches. The use of intestinal slices is attractive in biotransformation studies because these slices show a high drug transformation capacity. Further studies are necessary to further improve the morphology of incubated slices and to check whether these slices quantitatively correctly reflect the *in vivo* activity of the intestines in various species.